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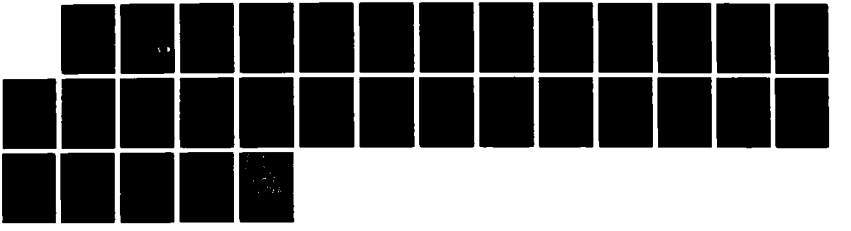

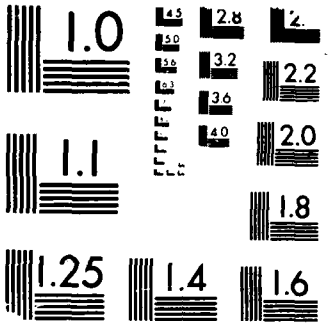

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STRUCTURE-FUNCTION RELATIONSHIP OF HYDROPHIIDAE POSTSYNAPTIC NEUROTOXINS

ANNUAL REPORT

ANTHONY T. TU

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This annual report is comprised of two main parts:

A. The major neurotoxin from the venom of Acalyptophis peronii captured in the Gulf of Thailand was isolated. Although there are two toxic fractions in the venom, the most toxic and abundant fraction was selected for purification and chemical characterization. The LD_{50} of the major toxin is 0.125 μg/g mice, indicating an extremely toxic nature. The toxin consists of 60 amino acid residues with methionine as the amino-terminal and asparagine as the carboxy-terminal end. It contains nine half-cystine residues. There is 1 mol each of tryptophan, tyrosine, methionine, valine, aspartic acid, leucine, and alanine, and there is no phenylalanine. The molecular weight calculated from the amino acid sequence determination was 6600. The toxin replaces α-bungarotoxin in binding with the acetylcholine receptor, indicating that the A. peronii major neurotoxin competes with α-bungarotoxin for the same binding site of the actylcholine receptor.

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Abstract (continued)

B. The acetylcholine receptor connects the nerve impulse from the axon to the muscle by receiving a nerve transmitter, acetylcholine. The receptor consists of five subunits of which two are identical; it is expressed as $\alpha_2\beta\gamma\delta$. It is known that the α subunit is the site for the acetylcholine binding and also for its antagonist, snake postsynaptic neurotoxin. It is generally recognized that the subunits β, γ, and δ are also essential to maintain the integrity of the acetylcholine receptor. In order to further understand the role of the subunits in the acetylcholine receptor function, the subunits were cross-linked with dimethyl suberimidate. The cross-linked acetylcholine receptor does not dissociate into its components and retains the binding activity to Lapemis toxin, a postsynaptic toxin from sea snake Lapemis hardwickii venom. This indicates that covalently linked acetylcholine receptor subunits retain their biological function as long as the neurotoxin binding site is not blocked.

FOREWORD

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

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1. Statement of the Problem Under Study

   A. There are 50 to 60 varieties of sea snakes, with each having a different color and appearance. How their neurotoxins differ among themselves has not been completely identified yet. In order to understand the structure-function relationship of sea snake neurotoxin, the amino acid sequence of the major neurotoxin from Acalyptophis peronii venom was established.

   B. Acetylcholine receptor is composed of 2α subunits and one subunit each of β, γ, and δ. It is of interest and also important to find the role of inter-subunits. However, as a first step to this goal, all subunits were cross-linked covalently. The neurotoxin binding activity of the cross-linked receptor was investigated.

2. Background and Review of Appropriate Literature

   A. Sea Snake Neurotoxins

      1) Isolation of Neurotoxins

         Sea snake venoms contain potent neurotoxins that bind almost irreversibly to the postsynaptic acetylcholine receptors. Neurotoxins are the most extensively studied proteins of all the components present in sea snake venoms. Sea snake toxins are basic proteins with isoelectric points of 9 to 10. They range in molecular weight from 6000 to 8000. Although the neurotoxin is the main fraction, other proteins with molecular weights up to 29,500 can be found in Pelamis platurus (Shipman and Pickwell, 1973).

         A number of postsynaptic neurotoxins have been isolated from a variety of sea snake venoms. Some of these toxins were isolated in the crystal form and were eventually used for X-ray crystallography (Uwatoko et al., 1966 ; Tamiya and Arai, 1966; Tu and Hong, 1971; Tu et al., 1971).

         Most sea snake neurotoxins consist of 60-62 amino acid residues with 4 disulfide bonds. These are the Type I or short chain neurotoxins. However, several Type II or long chain toxins were also isolated. Both Types I and II are postsynaptic toxins but Type II toxins have 5 disulfide bonds. Some neurotoxins have structures between Type I and II; they contain 4 disulfide bonds but have many other features

similar to Type II neurotoxins. Therefore, to classify neurotoxins solely on the basis of their disulfide bonds or amino acid sequences is an oversimplification.

2) Amino Acid Sequences of Neurotoxins

The amino acid sequences of many sea snake neurotoxins have been determined. As mentioned earlier, some neurotoxins have 4 disulfide bonds, while others have 5 disulfide bonds. These are the most commonly found neurotoxins in Hydrophiidae (sea snake) venoms and also in Elapidae venoms.

The amino acid sequences of Lc a and Lc b of Laticauda colubrina were unusual. They both consist of 69 amino acid residues with 4 disulfide bonds, yet their amino acid sequences were similar to those of type II (long chain) postsynaptic neurotoxins (Kim and Tamiya, 1982). Therefore, from the structural viewpoint, sea snake neurotoxins are of the following types:

	Disulfide bonds	SH	Example	Remark
Type I (short chain)	4	0	toxins 4.5	From E. schistosa venom
	4	1	lapemis toxin	From L. hardwickii venom
Type II (long chain)	5	0	LSIII	From Laticauda semifasciata venom
Hybrid type	4	0	Lc a	Amino acid sequence similar to Type II toxins
			Lc b	From L. colubrina venom

Sea snake neurotoxins show considerable homogeneity in their amino acid sequences. However, it is of interest to note that the amino acid sequences of the two subfamilies have some differences. Sea snakes within a subfamily usually have a greater degree of homology in their amino acid sequences with each other than with those from other subfamilies. There are many invarient

residues. The fact that there is greater similarity in sequences within a subfamily and more differences between the two subfamilies of sea snakes is very interesting since these chemical data agree well with morphological differences between the two subfamilies.

There are two subfamilies within the family Hydrophiidae. They are Hydrophiidae and Laticaudinae. The two types of sea snakes have distinct differences in their ventral scales. The former usually does not have ventral scales distinguishable from the surrounding scales, whereas the latter has wide ventral scales. These differences in ventral scale patterns eventually dictate their habitats. The reason a snake can crawl is due to the movement of its ventral scales. Since hydrophiinae do not have such ventral scales, they spend their entire lives in the sea. On the other hand, the Laticaudinae can swim in the sea and crawl on the beach and rocks.

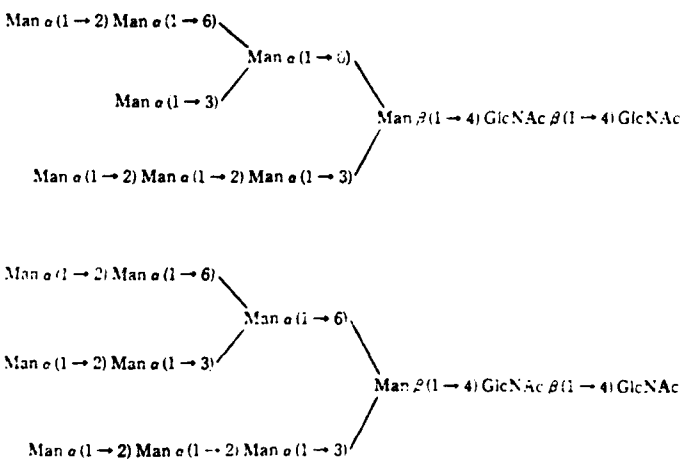
Despite great differences in the color patterns and appearance of different species of sea snakes, their venoms retain a high degree of homology in the amino acid sequences of the neurotoxins. Thus it is not surprising to see that conformations (protein backbone structure) of the neurotoxins are also similar. Similarity in the structures of sea snake neurotoxins are also reflected in immunological similarities.

B. Acetylcholine Receptor (ACR)

The origin of neurotoxicity by postsynaptic neurotoxins is due to their binding to acetylcholine receptors. It is, therefore, essential to understand the structure of the acetylcholine receptor and especially the neurotoxin binding sites in the acetylcholine receptor.

ACR is composed of subunits, but the composition of ACR depends on the species. The most common source of ACR is from Torpedo californica. It is composed of two α-subunits and one each unit of β, γ, and δ. Therefore, it is often expressed as $\alpha_2\beta\gamma\delta$. The ACR from calf is $\alpha\beta\gamma\delta\varepsilon$.

ACR is a glycoprotein and the carbohydrates attach to α, β, γ, and δ subunits. The structure of carbohydrate in each subunit is different (Nomoto et al., 1986). The most common carbohydrates are the following two types:



As to the binding site of ACR to neurotoxins, it has not been clarified completely. However, some useful information is available and is summarized in this section.

The synthetic dodecapeptide Lys-His-Trp-Val-Tyr-Tyr-Thr-Cys-Cys-Pro-Asp-Thr was found to bind to α-bungarotoxin (Neumann et al., 1986). This peptide sequence is actually the portion of the α subunit. It seems that ACR contains multiple binding sites. Evidence was presented using dendrotoxin showing stoichiometry of 4 toxins/ACR. On the other hand, two moles of α-cobrotoxin bound to one mole of ACR (Conti-Tronconi and Raftery, 1986). Proteolytic fragments of the α subunit bound to α-bungarotoxin (Wilson et al., 1984). Many ACR ligands were also known to bind to α subunits (Pederson et al., 1986; Ratnam et al., 1986; Oblas et al., 1986).

3. Rationale Used in Current Study

A. Acalyptophis peronii is the third most common sea snake in the Gulf of Thailand. Prior to this, a neurotoxin from A. peronii had not been isolated. In this investigation, the major neurotoxin was isolated from A. peronii venom and its complete amino acid sequence was identified.

In order to determine the nature of the neurotoxicity, the competing binding action of the toxin with α-bungarotoxin to acetylcholine receptor was also investigated.

B. Postsynaptic neurotoxin binds to acetylcholine receptor and blocks the binding of the neurotransmitter, acetylcholine. When acetylcholine binds to the receptor, the pore formed by five subunits enlarges and allows cations to pass through. By this mechanism, depolarization wave from the axon is transmitted to the muscle. If all subunits are connected by covalent bonds, the receptor would have less flexibility. It is of interest to examine how such cross-linked receptor binds to neurotoxins.

4. Experimental Methods

A. 1) Venom. Sea snake, A. peronii, was captured in the Gulf of Thailand, off the east coast of the Kra Isthmus. Venom was extracted from the venom glands after pulverization according to an earlier published method (Tu and Hong, 1971).

2) Isolation procedures. All of the purification procedures were performed at 4°C according to the method of Tu and Hong (1971).

3) Toxicity test. The toxicity tests were done by injecting 0.1 ml of venom of varying concentrations intravenously into Swiss white mice weighing 20 g each. At each of the seven dosage levels, eight mice were used. The number of mice which died was observed after 24 h. The toxicity was determined statistically by the method of Litchfield and Wilcoxon (1949) and expressed as the lethal dosage 50% (LD_{50}) value (micrograms of toxin per gram of body weight of mouse).

4) Homogeneity. The homogeneity of the toxin was checked using isotachophoresis and polyacrylamide gel electrophoresis. Capillary isotachophoresis was performed on a LKB Tachophor Model 2127 using a cationic system. The cationic system used 5 mM KOH adjusted to pH 7.0 with cacodylic acid as the leading electrolyte and creatinine (113 mg/100 ml) as the trailing electrolyte. Ampholine (1%), pH range 3.5 to 10, was used as a spacer ion. A constant current of 75 μA and a variable voltage of 3-12 kV were used. Polyacrylamide gel electrophoresis was done using the β-alanine acetate system as described previously (Tu et al., 1975).

5) Neurotoxin-acetylcholine receptor binding. Acetylcholine receptor (ACR) was isolated by the affinity chromatographic method of Froehner and Rafto (1979). Toxin binding to receptor was determined using the method of Schmidt and Raftery (1973).

The binding of the major toxin was measured by determining the decrease in ^{125}I-α-Bgtx binding to receptor following preincubation of the receptor with varying amounts of A. peronii toxin for 1 h. In other words, ^{125}I-α-Bgtx was used as a nonreversible back-titrant to measure the specific binding of a ligand competing for the same receptor (Fulpius et al., 1980, and Juillerat et al., 1982). Following preincubation of the receptor with varying amounts of A. peronii toxin, a saturating concentration of ^{125}I-α-Bgtx was added. This mixture was then incubated for 1 h, and the counts per minute determined as above.

6) Amino acid sequence. The purified major toxin was reduced and alkylated to make carboxymethylated toxin by following the method of Crestfield as described by Elzinga (1970). The toxin fragments were made by incubating toxin with trypsin (Sigma) for 24 h using an enzyme to substrate ratio of 1:20 (w:w). The mixture was then separated to peptide fragments by a HPLC column (reverse phase C-18). Endopeptidase arginine-C (Boehringer Mannheim) was also used to make different fragments, which were also separated by C-18 HPLC column.

Amino acid sequence was determined directly on an Applied Biosystems 470 A protein sequencer and PTH analyzer, on-line PTH amino acid separation system 120 A.

7) Raman spectroscopy. The Raman spectra were obtained by excitation with 514.5-nm line (Spectra Physics Model SP-164 argon ion laser) with a green interference filter. The spectra represent an average of 10 scans and were recorded using a Spex Ramalog 5 Raman spectrophotometer and a Spex SCAMP data acquisition processor. All spectra were obtained with solid protein samples and were recorded for S-H and S-S stretching vibrational regions with a 4 cm^{-1} spectra width resolution.

B. 1) Acetylcholine receptor isolation. Acetylcholine receptor was isolated from Torpedo californica, purchased from Pacific Bio-Marine Laboratories, by the method of Froehner and Rafto (1979). Affinity chromatography was used for the isolation using Naja naja atra toxin on Sephadex 4B. Major postsynaptic neurotoxin, lapemis toxin, was isolated from the venom of Lapemis hardwickii (Hardwick's sea snake) by the method of Tu and Hong (1971).

2) Cross-link. Dimethyl suberimidate was purchased from Pierce Chemical Co., Rockford, Il. Cross-linking was performed following the procedure of Davis and Stark (1970) and Hendon and Tu (1979) which involved reacting acetylcholine receptor at a concentration of 0.3 mg/ml with dimethyl superimidate at a concentration of 0.6 mg/ml at room temperature in 0.2 M triethanolamine, pH 8.15. The cross-linking reagent was added in three aliquots over a 2 hr period with slow stirring by a magnetic stirrer. After the addition of the last aliquot the reaction was allowed to proceed for 3 additional hrs and then dialyzed.

3) Acetylcholine receptor-neurotoxin binding. Neurotoxin-acetylcholine binding study was done following the method of Schmidt and Raftery (1973) and Ishizaki et al. (1983). The method was described in more detail in Section 4A(5).

5. Results

A. 1) Isolation. The major neurotoxin from A. peronii venom was isolated by a combination of gel filtration and CM-cellulose chromatography. Homogeneity of the toxin was established by two independent methods: discontinuous disk electrophoresis (Fig. 1B) and analytical isotachophoresis (Fig. 1C). In each case, a single band was observed. The LD_{50} of the neurotoxin in mice was found to be 0.125 µg/g by intravenous injection, indicating an extremely toxic nature of the neurotoxin. The LD_{50} of purified sea snake neurotoxin from Pelamis platurus by iv was 0.13 µg/g (Tu et al., 1975). A purified toxin from Hydrophis ornatus gave an LD_{50} value of 0.09 µg/g by intramuscular injection (Tamiya et al., 1983). Therefore, the toxicity of A. peronii neurotoxin is comparable to the toxicity of neurotoxins isolated from P. platurus and H. ornatus.

The yield of the major toxin from 1 g A. peronii venom was found to be 18.3 mg.

2) Amino acid sequence. The purified toxin was subjected directly to amino acid sequence analysis and the sequence of 37 residues from the amino terminal end was established.

Two proteolytic enzymes were used in order to obtain peptide fragments from the major toxin. Amino acid sequences of peptides from five

fractions were identified. Fraction 1 was a mixture of two peptides. One was G-T-I-I-E-R, corresponding to residues 32 to 37. The other fragment had a sequence of V-K-S-G partially overlapping with the sequence of fraction 5. Later V-K-S-G was identified to be residues from 44 to 47. Fraction 2's sequence corresponded to the numbers 13 to 24. Fractions 3 and 4 also partially overlapped with the sequences of the major toxin at 1 to 12 and 27 to 31. The amino acid sequence of fraction 5 was GCGCPQVKSGIK and was then subsequently identified as from numbers 38 to 49. The trypsin used apparently contained chymotrypsin, and this was responsible for the hydrolysis of W(27)-S(28) and Q(43)-V(44) resulting in fragments 4 and 1. The second endopeptidase used was endopeptidase arginine-C from the submaxillaris glands of mice (Fig. 2). The amino acid sequences from three fragments were identified (Fig. 3). The most important fragment was from fraction 3 and it gave the sequence of 32 to 60.

By combining all results of amino acid sequences of whole toxins and various fragments, a complete amino acid sequence of A. peronii neurotoxin was obtained (Fig. 3).

3) The toxin contains 1 mol each of tryptophan, tyrosine, valine, aspartic acid, leucine, and alanine and 9 mol of half-cystine. All short-chain snake postsynaptic neurotoxins contain four disulfide bonds. However, some toxins such as Lapemis toxin contain 1 mol of cysteine in addition to the four disulfide bonds. The presence of disulfide bonds and free cysteine should be detectable by Raman spectroscopy. Disulfide -S-S- has an intense stretching vibrational band at 508-540 cm^{-1}, while the free sulfhydryl group, -SH, shows the stretching vibrational band in the vicinity of 2570-2580 cm^{-1} (Tu, 1982; Parker, 1983; and Tu, 1986).

Raman spectra of A. peronii toxin in the ν_{S-S} ν_{S-H} (refers to stretching vibration in spectroscopy) regions indicate that indeed there are intense bands at 510 cm^{-1} ν_{S-S} and 2578 cm^{-1} for ν_{S-H} (Fig. 4). According to the recent investigations of Nash et al. (1985, 1986) and Sugeta et al. (1972, 1973), ν_{S-S} depends on the conformation of C-C-S-S-C-C networks. If the C-C-S-S-C-C has gauche-gauche-gauche conformation, the ν_{S-S} appears at 510 cm^{-1}, and trans-gauche-gauche conformation gives at 525 cm^{-1}, and

finally 540 cm^{-1} for trans-gauche-trans conformation. The analysis of other sea snake neurotoxins by Raman spectroscopy all showed that ν_{S-S} was in 510 cm^{-1}, indicating gauche-gauche-gauche conformation. Since four disulfide bonds are important in maintaining sea snake neurotoxins, the major toxin of A. peronii must also have similar overall conformation to other sea snake neurotoxins such as those from Lapemis hardwickii and Pelamis platurus (Yu et al., 1975 and Tu et al., 1976).

It is difficult to detect the sulfhydryl group by chemical methods, but it is easily detectable by Raman spectroscopy. For instance, a free sulfhydryl group was detected by Raman spectroscopy for TMV coat protein (2567 cm^{-1}) (Fox et al., 1979); Pelamis toxin (2578 cm^{-1}) (Tu et al., 1976); and lens protein (2582 cm^{-1}) (Kucks et al., 1976). A. peronii toxin showed the S-H stretching vibrational band at 2578 cm^{-1}, indicating the presence of a free sulfhydryl group.

It is, therefore, reasonable to assume that A. peronii toxin contains four disulfide bonds and one half-cystine just like the Lapemis toxin.

The pI was found to be higher than 9.5 using an ampholine range of 3.5 to 9.5.

Acetylcholine receptor binding. α-Bungarotoxin is a well-known postsynaptic neurotoxin that binds to ACR (Tu, 1973). In order to test whether A. peronii toxin can bind to ACR competitively with α-Bgtx, ^{125}I-α-Bgtx was used. If both α-Bgtx and A. peronii toxin bind to ACR but attach at different sites, the addition of A. peronii toxin in the presence of ^{125}I-α-Bgtx should not affect the binding of α-Bgtx to ACR. If this is the case, one expects to obtain the flat line seen in Fig. 5 instead of a downward curve. The experimental result indicates that as more A. peronii toxin is added, more α-Bgtx binding to ACR is diminished, as can be seen in decrease of cpm in Fig. 5. This indicates that both A. peronii toxin and α-Bgtx competitively bind to the same site of ACR. Usually the LD_{50} of neurotoxin is very low because of its high toxicity. A. peronii toxin shows the LD_{50} of 0.125 μg/g, which is in the range of other snake postsynaptic neurotoxin's LD_{50}.

B. SDS electrophoretic patterns of acetylcholine receptor before and after cross-linking are dramatically different (Fig. 6). The receptor without modification undergoes dissociation to individual subunits in SDS electrophoresis (Fig. 6-C). But the cross-linked receptor showed only one band without any trace of individual subunits (Fig. 6-B). Molecular weight range is shown using various proteins and it is shown in Fig. 1-A. The cross-linked acetylcholine receptor showed a very high molecular weight as it should. Figure 1 clearly shows the formation of high molecular cross-linked acetylcholine receptor.

In order to determine whether the cross-linked receptor can bind to a postsynaptic neurotoxin, lapemis toxin is iodinated with ^{125}I and the mutual binding was examined. Lapemis toxin without cross-linking was used as the positive control. The degree of binding for cross-linked receptor was almost the same as non-cross linked receptor (original receptor) as shown in Figs. 7 and 8. In Fig. 7, the effect of lapemis toxin concentration on acetylcholine receptor binding was shown and found to be linear. The effect of the receptor concentration on lapemis toxin binding was not entirely linear (Fig. 8).

6. Discussion

A. Sea snakes (Hydrophiidae) are subdivided into two subfamilies, Hydrophiinae and Laticaudinae. The amino acid sequence of purified toxins from Hydrophiinae, Laticaudinae, and land snake, Elapidae, were selected and compared (Fig. 9). The selection of toxin was somewhat arbitrary and based on random selection.

From Fig. 9, it is obvious that the closer the phylogenic relationship of the snakes, the more similar is the amino acid sequence. The amino acid sequences of neurotoxins within Hydrophiinae are very similar, and they are slightly different from Laticaudinae snake neurotoxins. The amino acid sequences of Hydrophiinae neurotoxins are more different from those of land snake Elapidae neurotoxins. By careful examination of the sequence of A. peronii major neurotoxin, it is very clear that it has the same sequence as toxins from H. ornatus and Astrotia stokesii (Tamiya et al., 1983; and Maeda and Tamiya, 1978). This is not unusual for many snake neurotoxins. The major neurotoxin of Lapemis hardwickii venom was identical to Enhydrina toxin (Fox et al., 1977; and Frykland et al., 1972).

There are two types of neurotoxins in snake venoms. One is presynaptic toxin and the other is postsynaptic toxin. The former inhibits the release of the neurotransmitter, acetylcholine, while the latter inhibits neurotransmission at the postsynaptic site of the motor end plate by binding to ACR. Presynaptic toxin does not bind to ACR. Since A. peronii toxin competitively binds to ACR with α-Bgtx, it is a postsynaptic-type neurotoxin. Similarity in primary structure to other well-known snake postsynaptic neurotoxins also supports the conclusion that A. peronii is a postsynaptic type toxin.

B. The acetylcholine receptor is composed of 2 α subunits, and one subunit each of β, γ, and δ. For the receptor to bind to the neurotoxin, all five subunits are necessary. This suggests that for toxin binding the assembly of all five subunits is essential. The interaction between each subunit is maintained by weak salt linkage. The crosslinking with dimethylsuberimidate produces a covalently linked subunit assembly. The covalently linked acetylcholine receptor is probably a more rigid assembly than the assembly of the natural receptor. The experiment shown in this report indicates that rigidity of subunit assembly within the receptor is not important as long as the toxin binding site in the α-subunit is available. The present report is the first one describing the covalently linked acetylcholine receptor assembly and its effect on toxin binding.

References

Conti-Tronconi, B. M., and Raftery, M. A. (1986) Proc. Natl. Acad. Sci. USA 83, 6646-6650.

Davis, G. E., and Stark, G. R. (1970) Proc. Natl. Acad. Sci. USA 66, 651-656.

Elzinga, M. (1970) Biochemistry 9, 1365-1374.

Fox, J. W., Elzinga, M., and Tu, A. T. (1977) FEBS Lett. 80, 217-220.

Fox, J. W., Lee, J., Amorese, D., and Tu, A. T. (1979) J. Appl. Biochem. 1, 336-343.

Froehner, S. C., and Rafto, S. (1979) Biochemistry 18, 301-307.

Fryklund, L., Eaker, D., and Karlsson, E. (1972) Biochemistry 11, 4628-4633.

Fulpius, B. W., Miskin, R., and Reich, E. (1980) Proc. Natl. Acad. Sci. USA 77, 4326-4330.

Hendon, R. A., and Tu, A. T. (1979) Biochim. Biophys. Acta 578, 243-252.

Ishizaki, H., Allen, M., and Tu, A. T. (1984) J. Pharm. Pharmacol. 36, 36-41.

Juillerat, M. A., Schwendimann, B., Havert, J., Fulpius, B. W., and Bargetzi, J. P. (1982) J. Biol. Chem. 257, 2901-2907.

Kim, H. S., and Tamiya, N. (1982) Biochem. J. 1, 215-223.

Kucks, J. F. R., East, E. J., and Yu, N.-T. (1976) Exp. Eye Res. 23, 9-14.

Litchfield, J. T., and Wilcoxon, F. (1949) J. Pharmacol. Exp. Ther. 96, 99-113.

Liu, C. S., and Blackwell, R. Q. (1974) Toxicon 12, 543-546.

Maeda, N., and Tamiya, N. (1974) Biochem. J. 141, 389-400.

Maeda, N., and Tamiya, N. (1976) Biochem. J. 153, 79-87.

Maeda, N., and Tamiya, N. (1978) Biochem. J. 175, 507-517.

Nash, C. P., Olmstead, M. M., Weiss-Lopez, B., and Musker, W. K. (1985) J. Amer. Chem. Soc. 107, 7194-7195.

Neumann, D., Barchan, D., Safran, A., Gershoni, J. M., and Fuchs, S. (1986) Proc. Natl. Acad. Sci. USA 83, 3008-3011.

Nomoto, H., Takahashi, N., Nagaki, Y., Endo, S., Arata, Y., and Hayashi, K. (1986) Eur. J. Biochem. 157, 233-242.

Oblas, B., Singer, R. H., and Boyd, N. D. (1986) Mol. Pharmacol. 29, 649-656.

Parker, F. S. (1983) Applications of Infrared, Raman, and Resonance Raman Spectroscopy in Biochemistry, Plenum, New York.

Pedersen, S. E., Dreyer, E. B., and Cohen, J. B. (1986) J. Biol. Chem. 261, 13735-13743.

Ratnam, M., Gullick, W., Spiess, J., Wan, K., Criado, M., and Lindstrom, J. (1986) Biochemistry 25, 4268-4275.

Schmidt, J., and Raftery, M. A. (1973) Anal. Biochem. 52, 349-354.

Shipman, W. H., and Pickwell, G. V. (1973) Toxicon 11, 375-377.

Strydom, A. J. C., and Botes, D. P. (1971) J. Biol. Chem. 246, 1341-1349.

Sugeta, H., Go, A., and Miyazawa, T. (1972) Chem. Lett., 83-86.

Sugeta, H., Go, A., and Miyazawa, T. (1973) Bull. Chem. Soc. Japan 46, 3407-3411.

Tamiya, N., and Arai, H. (1966) Biochem. J. 99, 624-630.

Tamiya, N., Maeda, N., and Cogger, H. G. (1983) Biochem. J. 213, 31-38.

Tu, A. T. (1973) Annu. Rev. Biochem. 43, 235-258.

Tu, A. T. (1982) Raman Spectroscopy in Biology, Principles and Applications, Wiley, New York.

Tu, A. T. (1986) in Spectroscopy of Biological Systems (Clark, R. J. H., and Hester, R. E., Eds.), pp. 47-112.

Tu, A. T., and Hong, B.-S. (1971) J. Biol. Chem. 246, 1012-1016.

Tu, A. T., Hong, B. S., and Solie, T. N. (1971) Biochemistry 10, 1295-1304.

Tu, A. T., Jo, B. H., and Yu, N.-T. (1976) Int. J. Peptide Protein Res. 8, 337-343.

Tu, A. T., Lin, T. S., and Bieber, A. L. (1975) Biochemistry 14, 3408-3413.

Uwatoko, Y., Nomura, Y., Kojima, K., and Obo, F. (1966) Acta Med. Univ. Kagoshima 8, 151-156.

Viljoen, C. C., and Botes, D. (1973) J. Biol. Chem. 248, 4915-4923.

Wilson, P. T., Gershoni, J. M., Hawrot, E., and Lentz, T. L. (1984) Proc. Natl. Acad. Sci. USA 81, 2553-2558.

Yang, C. C., Yang, H. J., and Huang, J. S. (1969) Biochim. Biophys. Acta 188, 65-77.

Yu, N.-T., Lin, T.-S., and Tu, A. T. (1975) J. Biol. Chem. 250, 1782-1785.

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Fig. 1 Isolation scheme of the major neurotoxin from Acalyptophis peronii venom (A and B). The isolation procedure is described under Materials and Methods. A single band of major toxin (2.5 μg) occurs when applied to polyacrylamide gel electrophoresis using a β-alanine acetate system (B) or analytical isotachophoresis, LKB Tachophor Model 2127, using a cationic system (C).

Fig. 2 HPLC peptide separation pattern of the carboxymethylated A. peronii major toxin after hydrolysis by endopeptidase Arg C (A). Peak 3 was rechromatographed under the same conditions before sequence determination (B).

Fig. 3 Amino acid sequence of the major toxin from Acalyptophis peronii venom. Arrows under the amino acids indicate the length of each Edman degradation experiment.

Fig. 4 Laser Raman spectrum of A. peronii major toxin (lyophilized powder) in the region of the S-S stretching vibration and the S-H stretching vibration.

Fig. 5 Binding of A. peronii major neurotoxin to the ACR binding site was measured as a decrease in ^{125}I-α-Bgtx binding following preincubation of ACR (2.0 μg) with varying amounts of A. peronii major neurotoxin.

Fig. 6 Electrophoresis of A. Standard proteins (66K, bovine albumin; 45 K, egg albumin; 36K, glyceraldehyde-3-phosphate dehydrogenase; 29K, carbonic anhydrase; 24K, trypsinogen). B. Cross-linked acetylcholine receptor (10 μg). C. Original acetylcholine receptor (10 μg). The unmodified receptor (C) undergoes dissociation to constituent subunits α, β, γ, and δ. The cross-linked receptor (B) showed very high molecular weight. The acetylcholine receptor and standards were treated with sample buffer containing 4% sodium dodecyl sulfate (SDS), 20% glycerol, and 10% 2-mercaptoethanol for 5 min at 100°C and then run on 10% polyacrylamide gel.

Fig. 7 The effect of neurotoxin concentration on acetylcholine receptor-neurotoxin binding. The binding of ^{125}I-Lapemis toxin to the acetylcholine receptor (3.0 μg) were assayed using DEAE disc. (o—·—o), Original acetylcholine receptor; (●—●), Cross-linked acetylcholine receptor; (x---x), Nonspecific binding of ^{125}I-Lapemis toxin to the acetylcholine receptor.

Fig. 8 The effect of acetylcholine receptor concentration on acetylcholine receptor-neurotoxin binding. (o—·—o), Original acetylcholine receptor; (●—●), Cross-linked acetylcholine receptor.

Fig. 9 Comparison of amino acid sequence studies of purified neurotoxins from Hydrophiidae and Elapidae families. Neurotoxins within the Hydrophiidae family have high sequences homologies. Low sequence homology is seen between families.

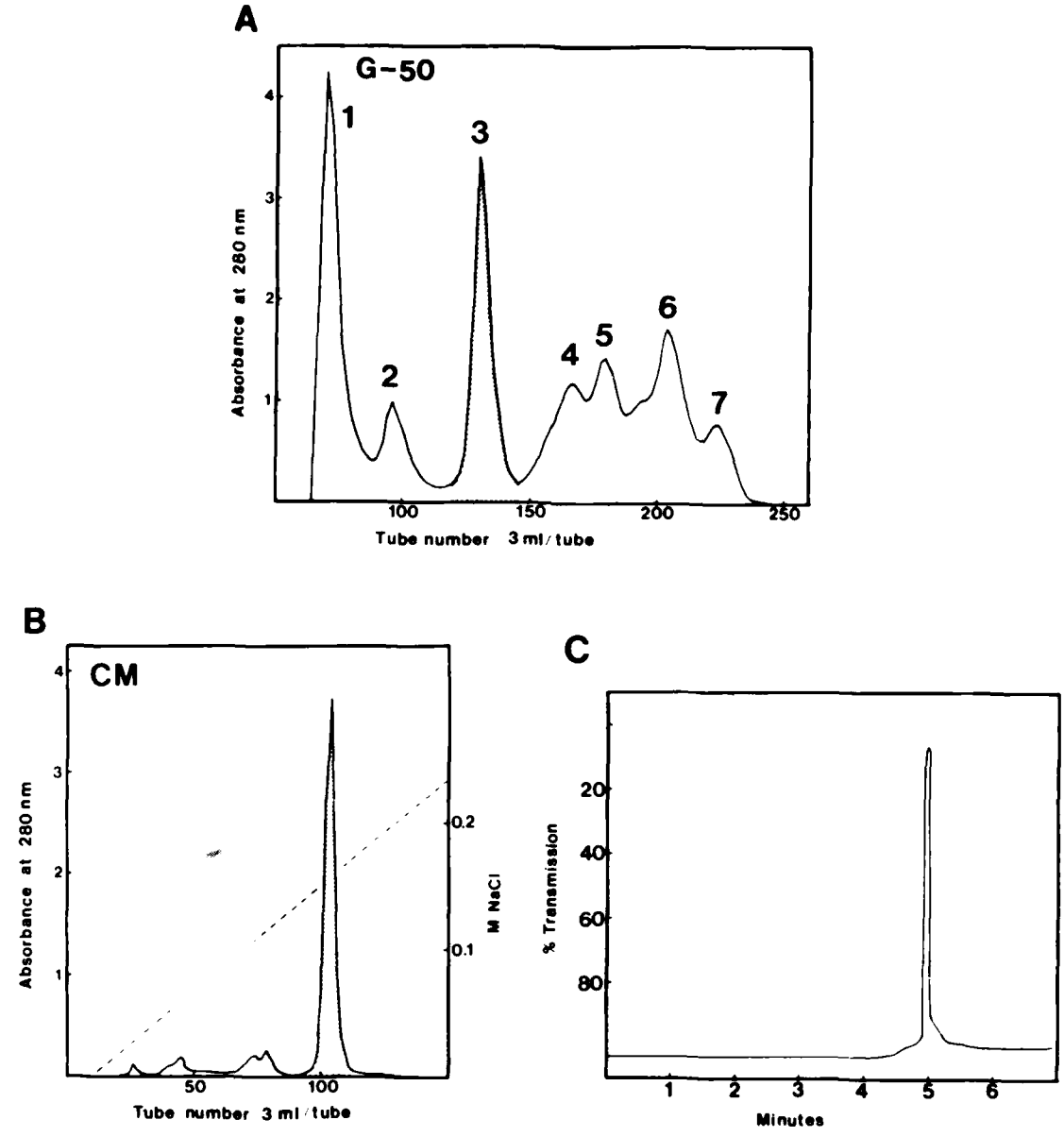
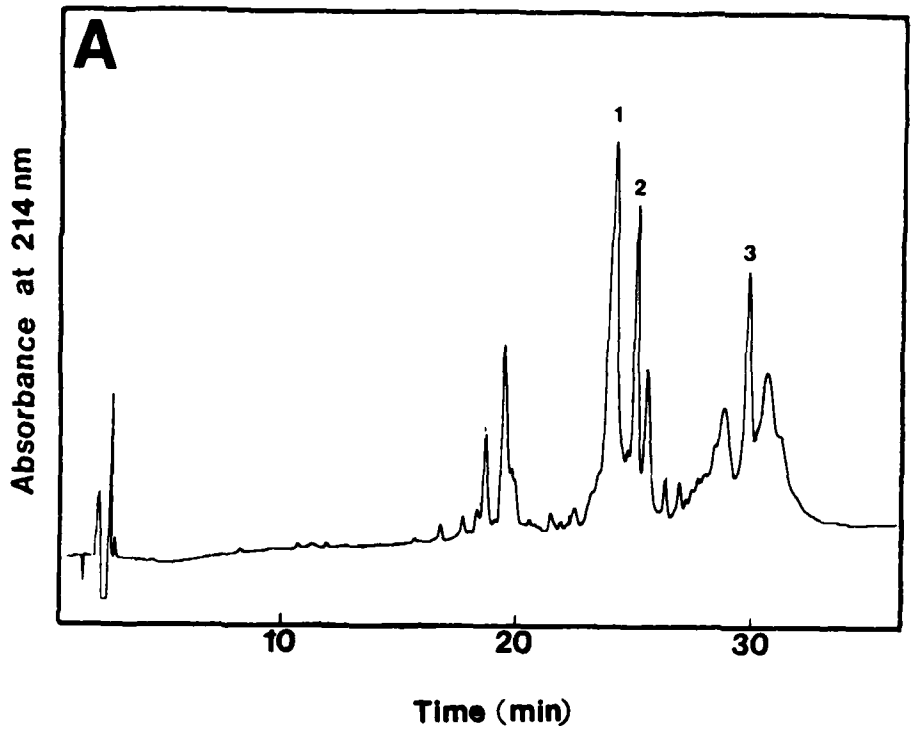

A
G-50
1
2
3
4
5
6
7
Absorbance at 280 nm
Tube number 3 ml/tube
B
CM
Absorbance at 280 nm
M NaCl
Tube number 3 ml/tube
C
% Transmission
Minutes

Fig. 1


A
Absorbance at 214 nm
1
2
3
10
20
30
Time (min)

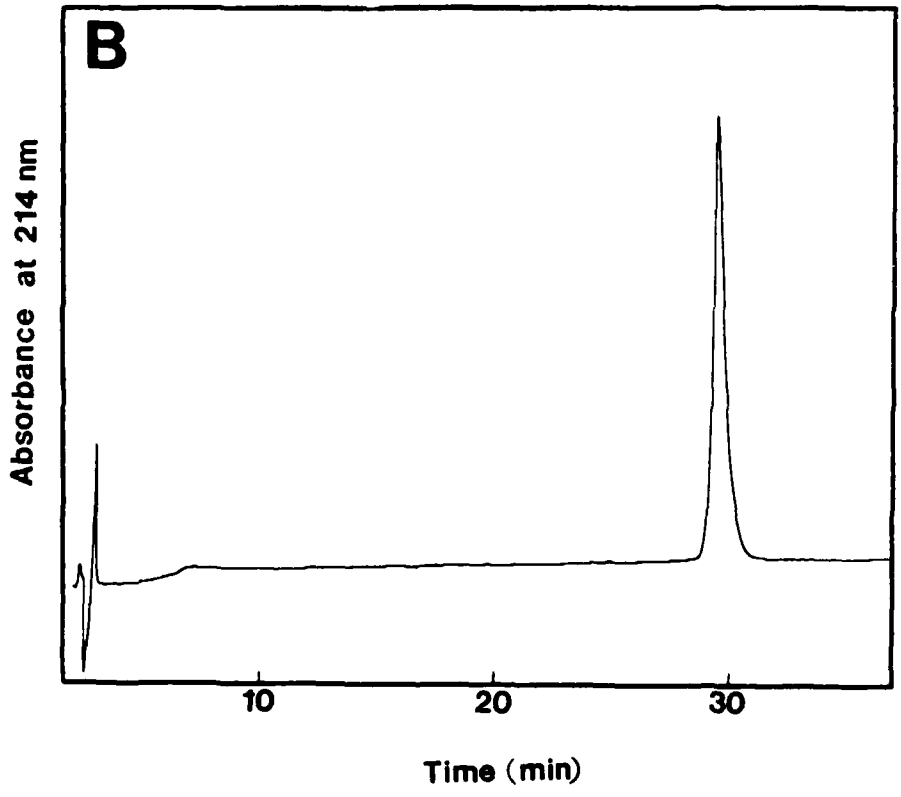

B
Absorbance at 214 nm
10
20
30
Time (min)

Fig. 2

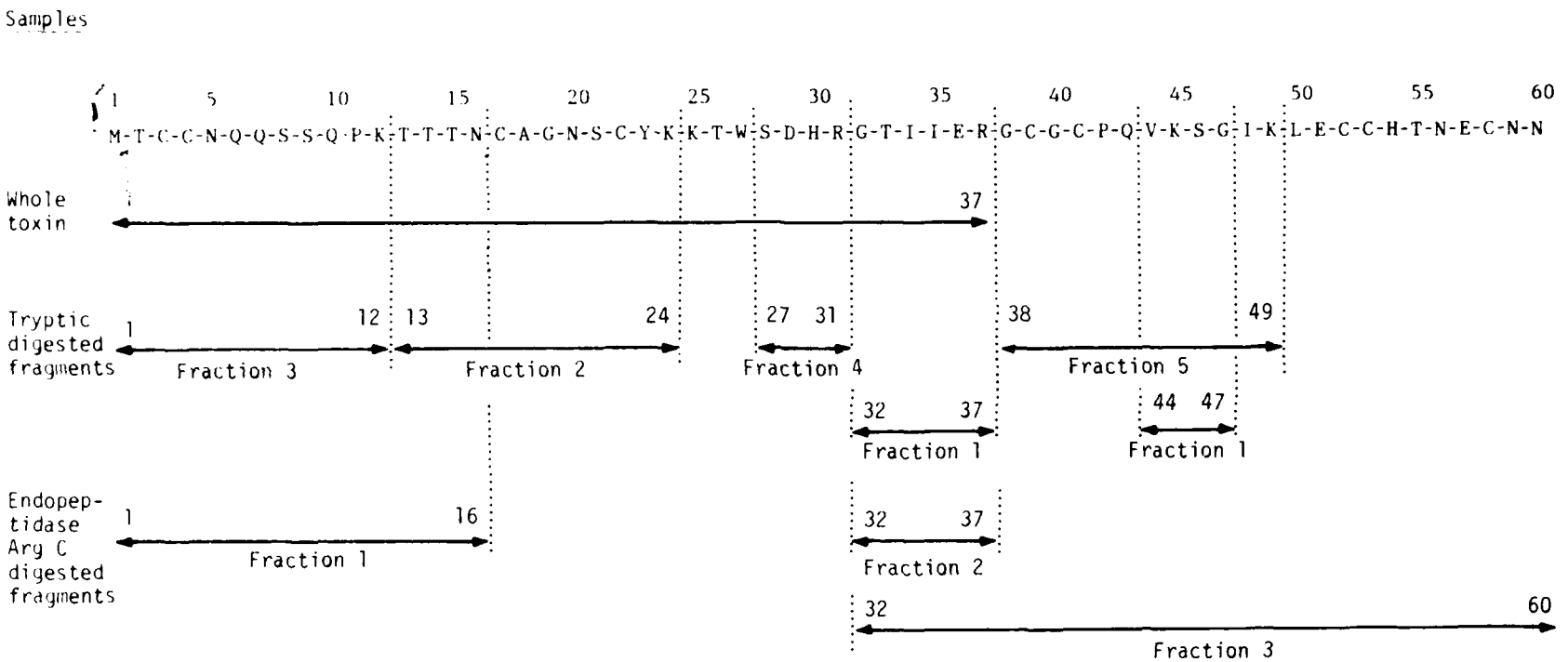

Samples
1 5 10 15 20 25 30 35 40 45 50 55 60
M-T-C-C-N-Q-Q-S-S-Q-P-K-T-T-T-N-C-A-G-N-S-C-Y-K-K-T-W-S-D-H-R-G-T-I-I-E-R-G-C-G-C-P-Q-V-K-S-G-I-K-L-E-C-C-H-T-N-E-C-N-N
Whole toxin
1
37
Tryptic digested fragments
1
12
13
24
27
31
38
49
Fraction 3
Fraction 2
Fraction 4
Fraction 5
32
37
44
47
Fraction 1
Fraction 1
Endopep-tidase Arg C digested fragments
1
16
32
37
Fraction 1
Fraction 2
32
60
Fraction 3

Fig. 3

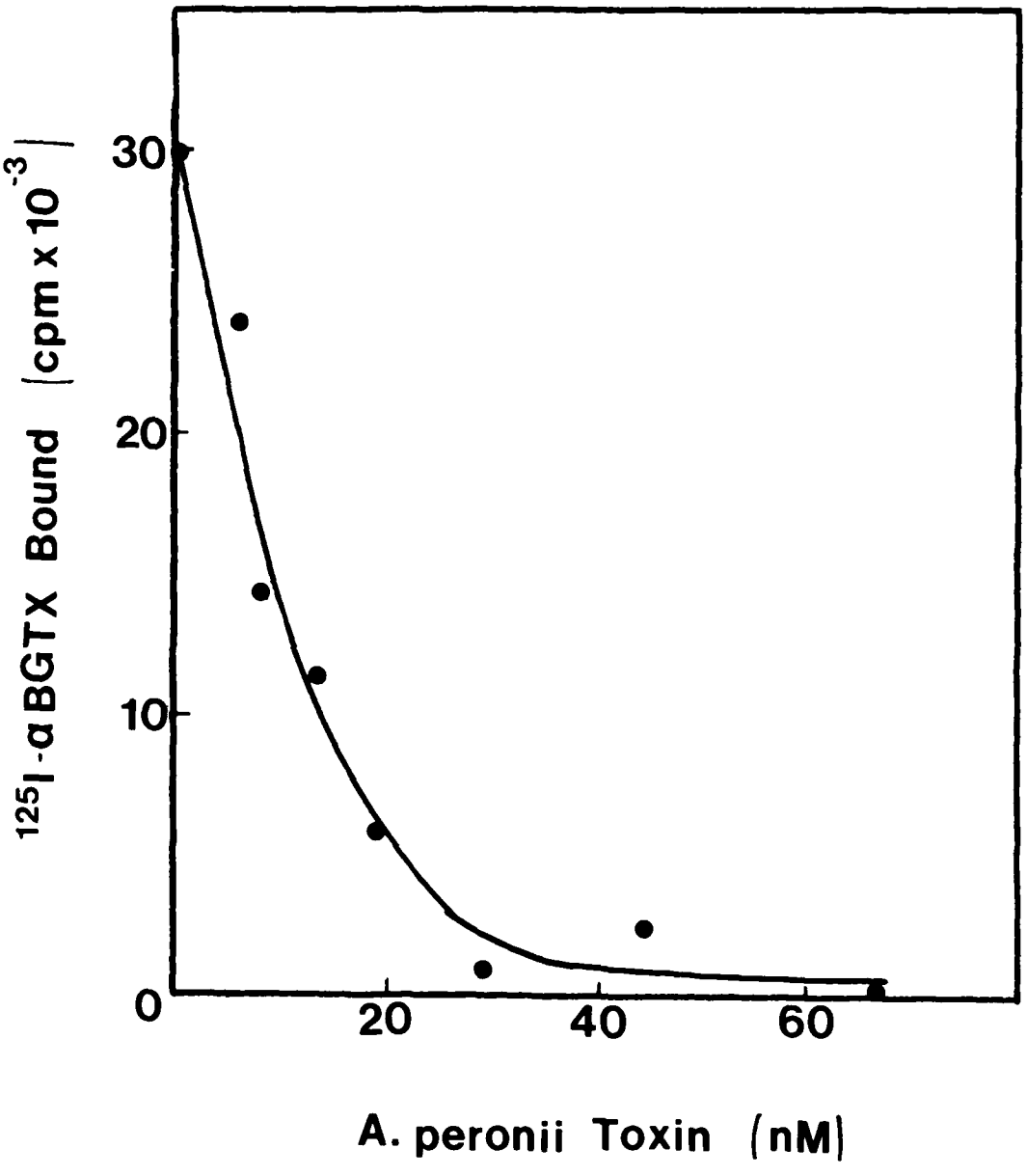


Fig. 4

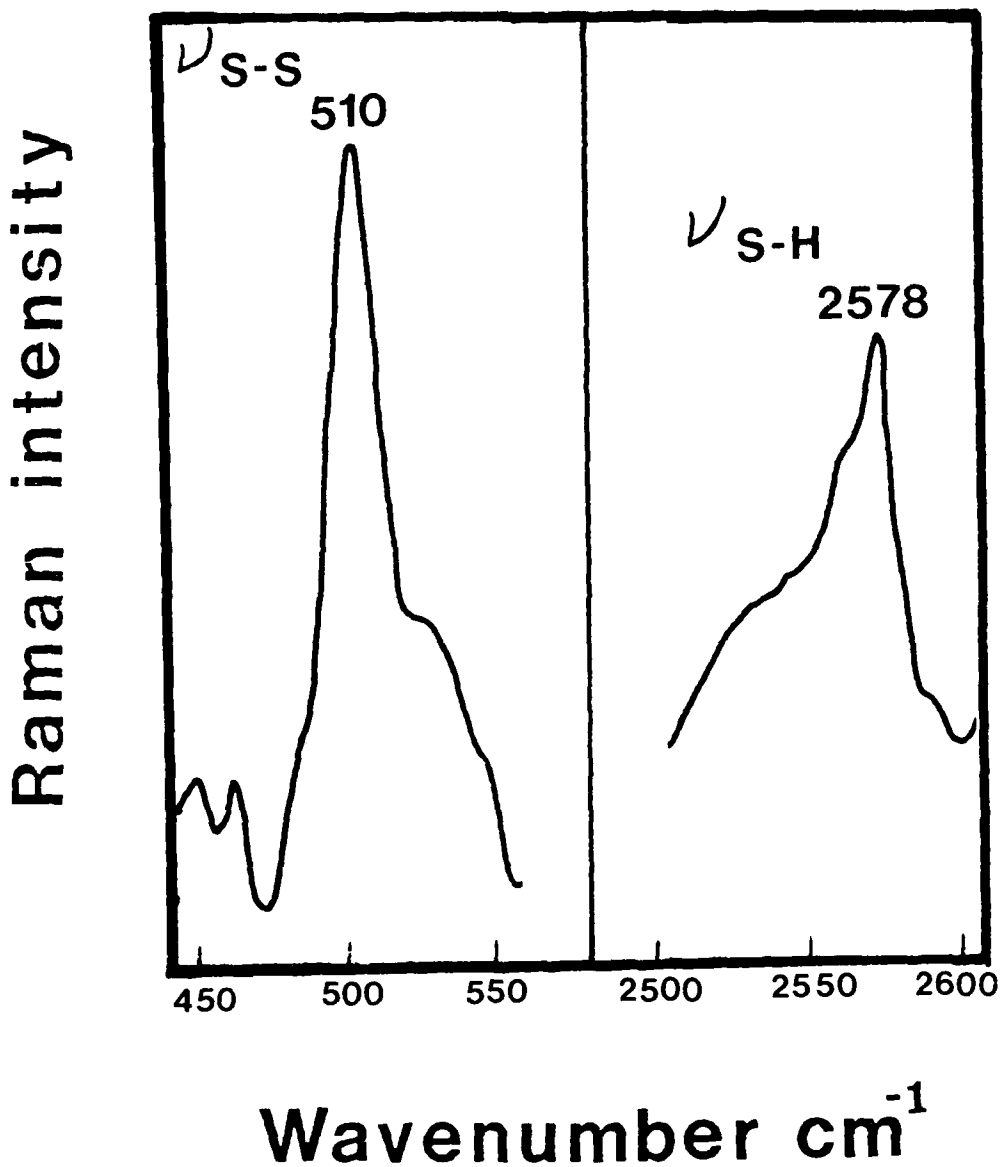

Raman intensity
ν_{S-S}
510
ν_{S-H}
2578
450
500
550
2500
2550
2600
Wavenumber cm^{-1}

Fig. 5

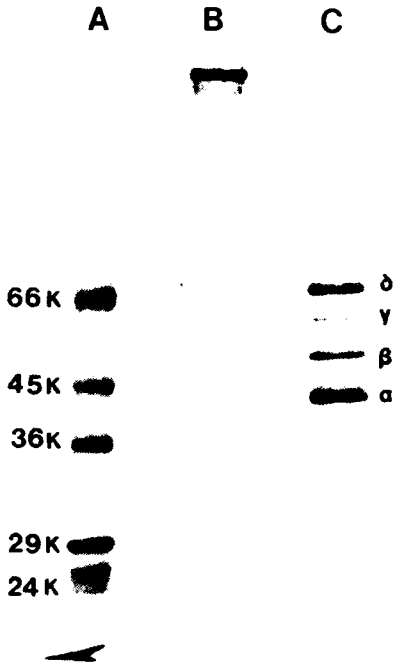


Fig. 6

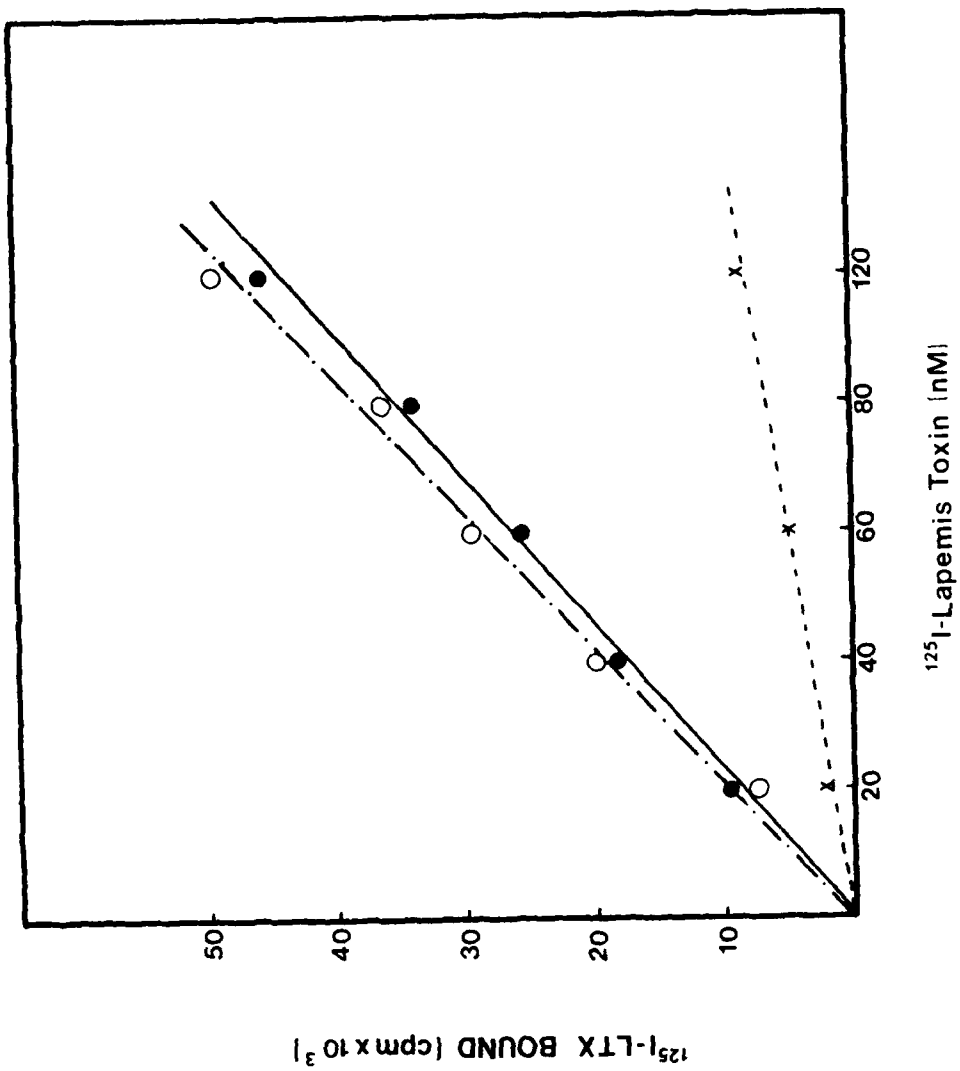

^{125}I-LTX BOUND (cpm x 10^3)
50
40
30
20
10
20
40
60
80
120
^{125}I-Lapemis Toxin (nM)

Fig. 7

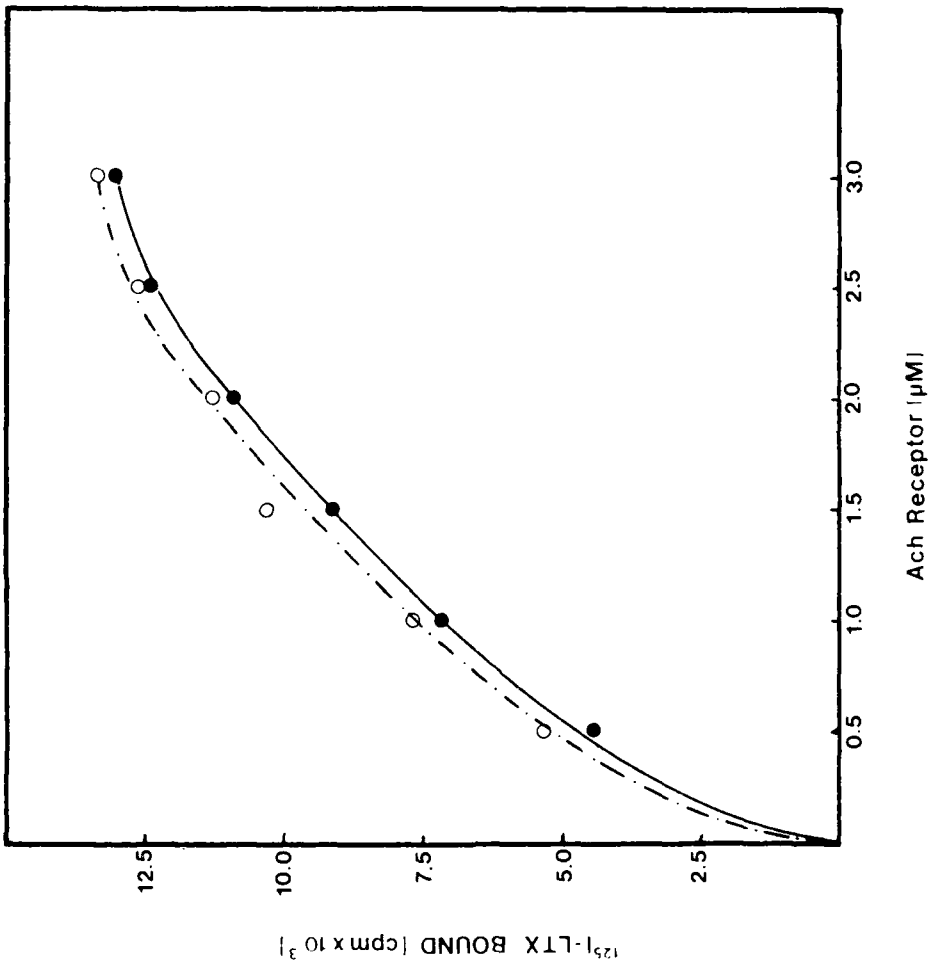

^{125}I-LTX BOUND (cpm x 10^3)
12.5
10.0
7.5
5.0
2.5
0.5
1.0
1.5
2.0
2.5
3.0
Ach Receptor (μM)

Fig. 8

Snake	Sequence	Reference
Family: Hydrophiidae		
Subfamily: Hydrophiinae	5 10 15 20 25 30 35 40 45 50 55 60	
Acalyptophis peronii	M T C C N Q Q S S Q P K T T T N C - A G - N S C Y K K T W S D H R G T I I E R G C G C P Q V K S G I K L E C C H T N E C N N	Present paper
Lapemis hardwickii	M T C C N Q Q S S Q P K T T T N C - - A E S S C Y K K T W S D H R G T R I E R G C G C P Q V K P G I K L E C C H T N E C N N	Fox et al. (1977)
Hydrophis cyanocinctus		
a	M T C C N Q Q S S Q P K T T T N C - - A E S S C Y K K T W S D H R G T R I E R G C G C P Q V K K G I K L E C C H T N E C N N	Liu and Blackwell (1974)
b	M T C C N Q Q S S Q P K T T T N C - - A E S S C Y K K T W S D H R G T R I E R G C G C P Q V K S G I K L E C C H T N E C N N	Liu and Blackwell (1974)
Subfamily: Laticaudinae		
Aipysurus laevis		
a	L T C C N Q Q S S Q P K T T T D C - - A D N S C Y K K T W Q D H R G T R I E R G C G C P Q V K P G I K L E C C K T N E C N N	Maeda and Tamiya (1976)
b	L T C C N Q Q S S Q P K T T T D C - - A D N S C Y K M T W R D H R G T R I E R G C G C P Q V K P G I K L E C C K T N E C N N	Maeda and Tamiya (1976)
c	L T C C N Q Q S S Q P K T T T D C - - A D N S C Y K K T W K D H R G T R I E R G C G C P Q V K P G I E L E C C K T N E C N N	Maeda and Tamiya (1976)
Laticauda laticaudata	R R C F N H P S S Q P Q T N K S C P P G E N S C Y N K Q W R D H R G T I T E R G C G C P T V K P G I K L T C C Q S E D C N N	Maeda and Tamiya (1974)
Family: Elapidae		
Naja naja atra	L E C H N Q Q S S Q T P T T T G C S G G E T N C Y K K R W R D H R G Y R T E R G C G C P S V K N G I E I N C C T T D R C N N	Yang et al. (1969)
Dendroaspis angusticeps	T M C Y S H T T T S R A I L T N C - - G E N S C Y R K S R R H P P K M V L G K G C G C P P G D D N L E V K C C T S P D K C N Y	Viljoen and Botes (1973)
Hemachatus haemachates		
II	L E C H N Q Q S S Q P P T T K S C P - G D T N C Y N K R W R D H R G T I I E R G C G C P T V K P G I N L K C C T T D R C N N	Strydom and Botes (1971)
IV	L E C H N Q Q S S Q T P T T Q T C P - G E T N C Y K K Q W S D H R G S R T E R G C G C P T V K P G I K L K C C T T D R C N K	Strydom and Botes (1971)

Fig. 9

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